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Induction of a Specific *N*-Methyltransferase Enzyme by Long-Term Heat Stress during Barley Leaf Growth[1]

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TIMOTHY J. LELAND[2] AND ANDREW D. HANSON*
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Previous work showed that the indole alkaloid gramine accumulates in the upper leaves (*e.g.* the fifth) of barley as a response to high growth temperatures. The biosynthesis of gramine proceeds from tryptophan to 3-aminomethylindole (AMI); sequential *N*-methylations of AMI then yield *N*-methyl-3-aminomethylindole (MAMI) and gramine.

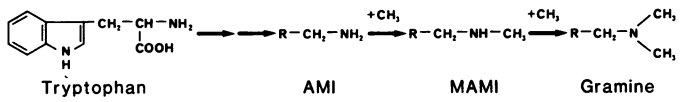
To determine whether high-temperature stress increases the activity of gramine pathway enzymes, leaf tissue from plants grown at various temperatures was assayed for *N*-methyltransferase (NMT) activity using AMI and MAMI as substrates in both *in vivo* and *in vitro* assays. NMT activity in expanding fifth leaves was increased 8- to 20-fold by growth at high temperatures (35°C day/30°C night) compared to cool temperatures (15°C/10°C). Several days of high temperature were required for full induction of NMT activity. No induction of NMT activity occurred in leaves which had completed expansion in cool conditions before exposure to high temperature.

To investigate NMT induction at the protein level, NMT activity was purified to homogeneity and used to produce polyclonal antibodies. Throughout enzyme purification, relative NMT activities towards AMI and MAMI remained constant, consistent with a single NMT enzyme. Immunoblot analysis showed that a large increase in NMT polypeptide coincided with induction of NMT activity by heat stress. Our results point to a type of high-temperature regulation of gene expression that is quite distinct from heat shock.

Gramine is a simple indole alkaloid found in the shoots of many barley (*Hordeum vulgare* L.) cultivars and wild barley lines (3, 12). Gramine biosynthesis involves the steps shown in Scheme 1. The indole nucleus and the methylene side chain of tryptophan are incorporated into the first stable intermediate of the pathway, AMI[3] (7, 9). AMI is then methylated at the amino nitrogen to form the secondary amine, MAMI, which is in turn *N*-methylated to produce the tertiary amine, gramine (7, 18, 22). Indirect evidence indicates that these methylations are catalyzed by an NMT enzyme (or enzymes) specific to the gramine pathway (11), for which SAM acts as the methyl donor (18). Although degradative pathways for gramine are known (6), gramine catabolism is very slow (11) so that accumulation is controlled mainly by the rate of synthesis.

Some barley cultivars are gramine free (11, 12). In the case of cv Proctor, the synthesis pathway is known to be blocked prior to AMI, because Proctor leaf tissue contains no AMI but can methylate supplied AMI and MAMI as actively as cultivars which contain gramine (11).

A



Scheme 1

Accumulation of gramine in barley is subject to both developmental and environmental control (11, 12). Developmental control is evident from a short phase of constitutive accumulation of gramine in young plants, particularly in the first leaf (11, 22). In plants grown at or below optimal temperatures, each successive leaf contains less gramine so that alkaloid levels are negligible in the fifth to tenth leaves (8, 11, 22). Gramine accumulation in these upper leaves can be elicited by growth at supra-optimal temperatures (11, 12). Only those leaves which actually emerge during the exposure to high temperature accumulate the alkaloid; previously expanded leaves do not (11).

Here, we establish by *in vivo* and *in vitro* assay methods that a large increase in activity of a specific NMT enzyme accompanies the induction of gramine accumulation by heat-stress. Using antibodies directed against purified NMT protein, we also demonstrate that high temperature induction of NMT activity is paralleled by an increase in NMT protein level.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Sources for the spring barley cultivars Proctor (CI 11806) and Arimar (CI 13626) were as given previously (12). Plants were grown in pots of soil mix (12) and irrigated with half-strength Hoagland solution. Standard growth chamber conditions were: d, 16 h, 200 μmol photon m^{-2} s^{-1} photosynthetic photon flux density, vapor pressure deficit 10 mbar; night, 8 h. For growth temperature experiments on the fifth leaf, plants were grown for the first 10 d at 15°C/10°C or 21°C/16°C (day/night) and thinned to two or three per pot. Some pots were then transferred to higher temperature regimes (30°C/25°C, 33°C/28°C, 35°C/30°C) and the experiment was continued until the fifth leaf was at the half-emerged stage, at which time it was harvested. The time between transfer and harvest varied according to temperature and cultivar. To obtain etiolated first leaves for enzyme characterization and purification, seedlings of cv Proctor were grown at 25°C for 6 to 8 d in darkness in flats of vermiculite.

Radiochemicals and Alkaloid Precursors. [^{14}C]Formate (57 or 59 μCi μmol^{-1}) and [^{14}C]SAM (59 or 60 μCi μmol^{-1}) were from

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[2] Present address: Funk Seeds International, 1300 West Washington, P. O. Box 2911, Bloomington, IL 61701.

[3] Abbreviations: AMI, 3-aminomethylindole; MAMI, *N*-methyl-3-aminomethylindole; [^{14}C]SAM, *S*-adenosyl-L-[methyl-^{14}C]methionine; NMT, *N*-methyltransferase; HSP, heat shock protein.

Table I. *Incorporation of ^{14}C from [^{14}C]Formate into Indole Alkaloids by Segments of Fifth Leaves from Plants Grown at 21°C/16°C and 30°C/25°C*

Plants were grown in optimal conditions (21°C/16°C) for 10 d; one-half were then transferred to mild heat-stress (30°C/25°C). Fifth leaves were harvested after a further 12 to 14 d, as they reached half-emergence. [^{14}C]Alkaloid synthesis was assayed at 21°C/16°C.

Experiment	Cultivar	Methyl Acceptor Supplied	^{14}C-Incorporation into Indole Aklaloids[a]		Increase
			21°C/16°C	30°C/25°C	
			nCi/3 segments		*-fold*
1	Arimar	None	15.6	55.9	3.6
	Proctor	None	<0.2	<0.3	
		AMI	20.2	72.1	3.6
		MAMI	10.4	32.0	3.1
2	Arimar	None	7.2	73.1	10.1
	Proctor	AMI	14.5	96.5	6.7
		MAMI	10.2	41.0	4.0

[a] Chromatography showed that for Proctor leaf segments supplied AMI, ^{14}C was present in MAMI (≥60%) and gramine (≤40%); for Proctor segments supplied MAMI, all ^{14}C was in gramine. Arimar segments contained mainly [^{14}C]gramine.

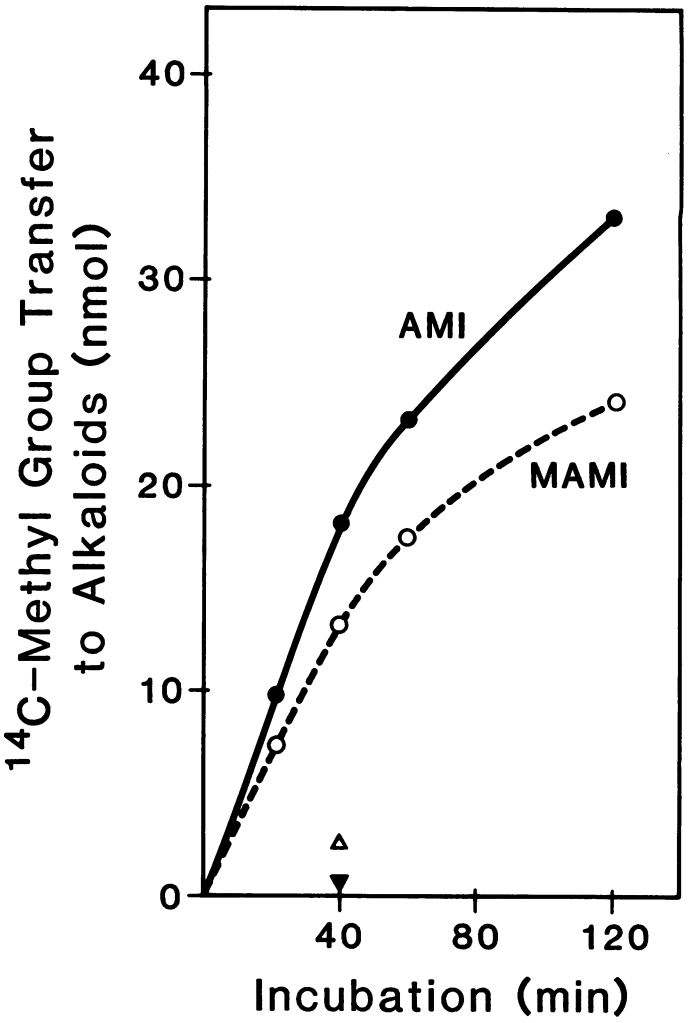

FIG. 1. Progress curves for ^{14}C-methylation of AMI (●) and MAMI (○) by extracts of dark-grown Proctor barley first leaves. Assay temperature was 25°C; assays contained 120 nmol [^{14}C]SAM, 600 nmol of methyl acceptor, and extract equivalent to 50 mg fresh weight of leaf. Samples without AMI or MAMI, or with boiled extract, showed no activity. Individual points at 40 min are results for tryptamine (▼) and tyramine (△) methyl acceptors. All data are means of duplicate samples. The experiments were repeated, with similar results.

Amersham Corp. The radiochemical purity of [^{14}C]SAM was checked by TLC on silica gel G developed with *n*-butanol:1M HCl:ethanol (5:5:2). [^{14}C]Gramine (0.16 or 0.20 μCi μmol^{-1}) and [^{14}C]MAMI (0.19 or 0.25 μCi μmol^{-1}) were isolated from Arimar first leaves fed [^{14}C[formate (11).

Gramine (Sigma) was recrystallized from acetone. Tryptamine HCl (NBC) and tyramine (Sigma) were recrystallized from 96% ethanol and checked for purity by paper chromatography in the 'Isobuff' system (11). AMI was synthesized according to Putochin (19) and Schallenberg and Meyer (20). MAMI was synthesized according to Gower and Leete (7). Both AMI and MAMI were purified on a Sephadex LH-20 column eluted with 50% (v/v) methanol in water. Confirmation of the identify of AMI and MAMI was obtained with a Hewlett-Packard 5985 quadrupole mass spectrometer by direct probe, as well as by TLC and paper chromatography with known standards. The *p*-dimethylaminocinnamaldehyde spray reagent was used to visualize indole compounds (11).

***In Vivo* Assay of NMT Activity.** This assay used [^{14}C]formate to label pools of methyl groups *in vivo* (11). For growth-temperature experiments, one or two 1-cm segments were cut from the basal furled portions of half-expanded fifth leaves. Batches of three segments were first infiltrated either with 1 μl/segment of unlabeled gramine precursor (AMI or MAMI, 9 nmol/segment) dissolved in K-phosphate buffer (20 mM, pH 7), or with buffer alone. After 1.5 to 3 h incubation on moist filter paper in darkness, segments were infiltrated with 1 μl/segment of [^{14}C] formate in H_2O (0.4–0.5 μCi/segment). Incubation was then continued for 24 h, in the 21°C/16°C growth chamber. Following the 24-h incubation, segments were frozen in liquid N_2; 100 mg of freeze-dried, ground 7-d Arimar shoots were added to each three-segment sample to provide carrier for ^{14}C-alkaloids during extraction. Alkaloids were extracted as described previously (12). Representative unlabeled samples were spiked with a small quantity of [^{14}C]gramine (4.0 nCi) or [^{14}C]MAMI (2.5 nCi), for estimation of gramine and MAMI recovery which averaged 65 and 56%, respectively. All results have been corrected for alkaloid recovery. Alkaloid fractions were analyzed in two or more of the following systems: TLC on silica gel G in methanol:acetone:concentrated HCl (90:10:4, v/v), or *n*-butanol: ethanol:concentrated NH_4OH (40:2:3, v/v), or chloroform: methanol:concentrated NH_4OH (80:15:1, v/v) (17); or paper chromatography in the 'Isobuff' system. Radioactive zones were located by autoradiography and eluted for scintillation counting. Identification of labeled alkaloids was based on co-chromatography with authentic standards.

***In Vitro* Assay of NMT Activity.** Half-expanded fifth leaves were harvested; in some cases second leaves were also taken. Leaves were ground at 4°C in a mortar and pestle with acid-washed sand in 50 mM Tris/HCl (pH 8.5) containing 7 mM DTT (2 ml/g fresh weight). Homogenates were centrifuged at 25,000*g* for 15 min and the supernatant (crude extract) was used for assays.

The assay was similar to those of Mudd (18), Mack and Slaytor (15) and Meyer (16). The standard assay mixture contained 150 mM glycylglycine/NaOH (pH 9.0), 5mM DTT, 0.6 mM [^{14}C]SAM (45 nCi μmol^{-1}, 5.4 nCi), and 3 mM AMI or MAMI combined with 150 μl of crude extract in a total volume of 200 μl. After a 30-min incubation at 25°C in a shaking water-bath, 500 nmol each of MAMI and/or gramine were added as carriers for labeled alkaloids, and the reaction was stopped with 0.20 ml of 1 M

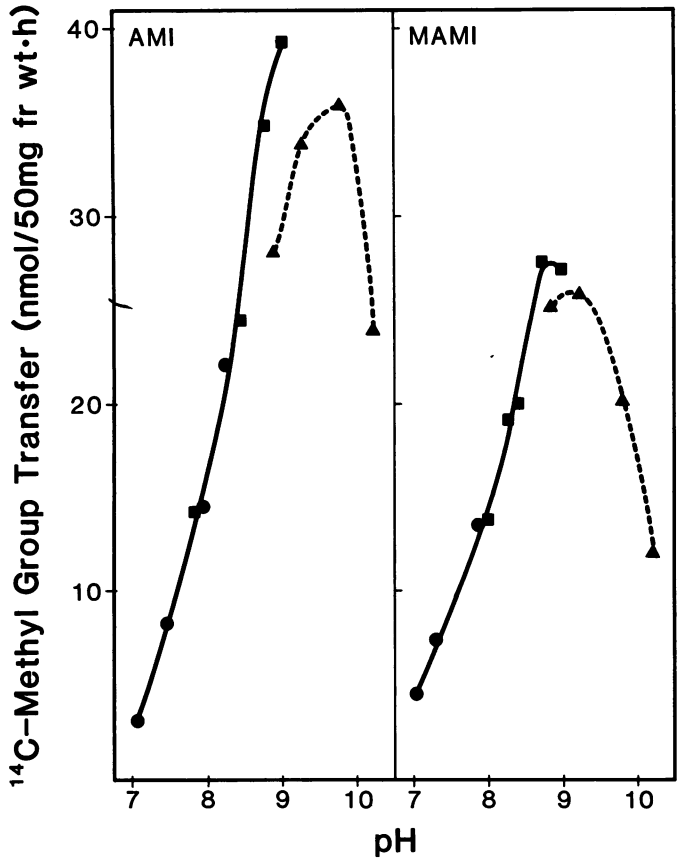

FIG. 2. Effect of pH on [^{14}C]SAM-dependent methylation of AMI (left) and MAMI (right) by extracts of dark-grown Proctor barley first leaves. Incubation was for 30 min. Buffers (150 mM) were: (●), Hepes/NaOH; (■), glycylglycine/NaOH; (▲), glycine/NaOH. Plotted pH values were actual measurements of complete assays. Data points are means of duplicate samples. The experiment was repeated, with similar results.

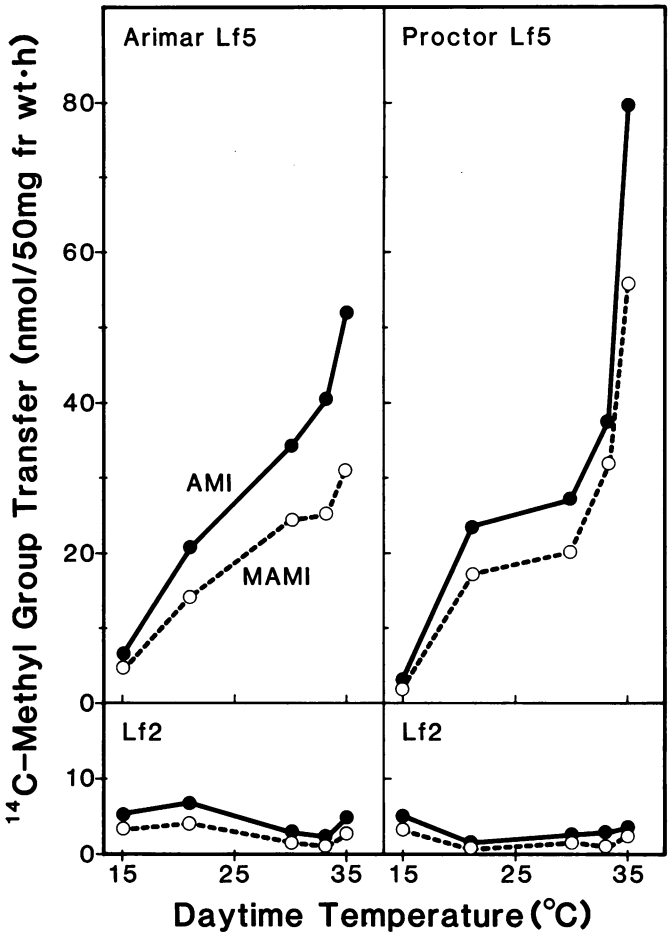

FIG. 3. Effect of growth temperature on *in vitro* NMT activity towards AMI (●) and MAMI (○), for barley cultivars Arimar (left) and Proctor (right). Plants were grown for 10 d in optimal temperature conditions (21°C/16°C) and then transferred to the various temperature regimes; night temperatures were 5°C below day temperatures. Upper frames are results for leaf 5, which accumulates alkaloids when plants are grown at high temperature. Lower frames are results for leaf 2, in which alkaloids do not accumulate at any temperature. Data points are means of four replicates. The experiment was repeated twice, with similar results.

H_3BO_3/1 M Na_2CO_3 buffer (pH 10). Alkaloids were extracted into 2 ml of $CHCl_3$, 1 ml of which was taken for scintillation counting. The remaining 1 ml was reduced to dryness in an N_2 stream and the residue was then redissolved in 60 mM HCl for chromatographic analysis of labeled products as described above. In blank assays, either without enzyme solution or without AMI/MAMI substrates, partitioning of ^{14}C into the $CHCl_3$ phase was always negligible. To estimate recovery of labeled alkaloids, representative unlabeled reaction mixtures were spiked with [^{14}C]gramine (2 nCi) or [^{14}C]MAMI (1.2 nCi). Recoveries averaged 90% for [^{14}C]gramine and 70% for [^{14}C]MAMI. Reported values have been corrected for recovery.

Enzyme Purification. Protein was determined according to Bradford (2) using BSA as a standard. All operations were carried out at 4°C. Buffer pH refers to the value at 4°C. The following procedure gave the highest specific activity purified product. Six- to 8-d old Proctor shoots (70 g fresh weight) were ground in a mortar and pestle with acid-washed sand in 70 ml of 50 mM Tris/HCl (pH 8.5) containing 10 mM DTT. The homogenate was centrifuged at 145,000*g* for 1 h; the supernatant was concentrated to about 8 ml (8–12 h) in an Amicon ultrafiltration cell with a PM-30 membrane; after adding 25 ml of 25 mM histidine/HCl (pH 6.2) containing 5 mM DTT, the sample was reconcentrated to about 7 ml. This concentrate was applied to a PBE 94 (Pharmacia) chromatofocusing column (1.5 × 30 cm) equilibrated with 25 mM histidine/HCl (pH 6.2). The column was eluted with Polybuffer 74 (Pharmacia, pH 5.0), at a flow rate of 20 ml h^{-1}. To reduce losses of NMT activity at low pH, fractions

Table II. *NMT Activity in Fifth Leaves Exposed to High Temperature for Various Times*

Plants were grown at 15°C/10°C and transferred to high temperature conditions for various times. Pulse (6 or 10 h) high temperature treatments were given in daytime growth chamber conditions; plants were then returned to 15°C/10°C. Fifth leaves were harvested when half-emerged.

Treatment	NMT Activity[a]	
	Proctor	Arimar
	nmol/50 mg fresh wt·h	
Continuous 15°C/10°C	3.1	6.1
Final 14 d at 33°C/28°C	48.1	60.5
Final 3 d at 33°C/28°C	9.3	20.4
6-h, 34°C pulse 4 d before harvest	8.9	
10-h, 34°C pulse 1 d before harvest	8.9	

[a] Assayed with AMI methyl acceptor. Data are means of duplicates.

Table III. *Purification of N-Methyltransferase from 8 d Etiolated Proctor Shoots*

Fraction	Volume	Total Protein	Total Activity[a]	Specific Activity	Recovery	Purification
	ml	*mg*	*units*[b]	*units/mg protein*	%	*-fold*
Crude extract	118	171	42,900	251		
Chromatofocusing	36	5.22	12,100	2,320	28.2	9.2
1st DEAE-HPLC (Bio-Sil TSK)	8	1.57	8,560	5,450	20.0	21.8
Gel filtration (Bio-Gel TSK)	7	0.228	3,030	13,300	7.0	52.9
2nd DEAE-HPLC	2	0.033	1,840	55,800	4.3	223

[a] Assayed with AMI substrate; activities with MAMI substrate were in the range 0.67 to 0.77 × those with AMI substrate for all fractions. [b] One unit of activity = incorporation of 1 nmol [^{14}C-methyl] into alkaloids per h.

(4 ml) were collected in tubes containing 0.2 ml of 2 M Tris/HCl (pH 7.8), plus 10 mM β-mercaptoethanol. Active fractions were combined, concentrated using PM-30 Centricon concentrators (Amicon), and injected onto a HPLC DEAE Bio-Gel TSK (75 × 7.5 mm) column. The column was eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM Tris/HCl, 5 mM DTT (pH 7.5), at a flow rate of 60 ml h^{-1}; the gradient required 40 min to complete. Active fractions were pooled, concentrated using a PM-30 Centricon, and loaded onto the first of two tandemly arranged HPLC gel filtration columns (Bio-Sil TSK-125, followed by Bio-Sil TSK-250, both columns 300 × 7.5 mm, Bio-Rad), equilibrated in 20 mM Tris/HCl, 100 mM Na_2SO_4, and 2 mM DTT (pH 7.2). Elution was at 30 ml h^{-1}. Active fractions were pooled and concentrated as before and carried through a second DEAE step as detailed above. Purified NMT protein was stored at −60°C in 20 mM Tris/HCl (pH 8.0), containing 5 mM DTT and 50% (v/v) glycerol.

Mol Wt Determination. Mol wt was determined either by HPLC gel filtration with Bio-Sil TSK-125 and TSK-250 columns in tandem using thyroglobulin, gamma globulin, ovalbumin, myoglobulin, and cyanocobalamin (Sigma) as standards or by SDS-PAGE using BSA, ovalbumin, glyceraldehyde-3-P dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor and α-lactalbumin (Sigma) as mol wt markers. SDS-PAGE was carried out in 1.5-mm thick slab gels according to Laemmli (14), with a separating gel of 13% polyacrylamide. Protein bands were stained with Coomassie brilliant blue R-250.

Production of Rabbit Immune Serum. Purified NMT protein was used to immunize a rabbit. Two immunizations, 18 d apart, were made with 150 μg protein emulsified in complete Freund's adjuvant. A final injection of 50 μg of protein in incomplete Freund's adjuvant was made 10 d later; serum was collected 8 d after the final injection.

Immunoblots. Samples of crude extract from fifth leaves (50 μg of soluble protein) were separated by SDS-PAGE as described above, along with purified NMT protein standards. At the end of a run, gels were placed in cold 25 mM Tris/192 mM glycine (pH 8.3) containing 20% (v/v) methanol (Towbin buffer; 23) for 20 to 30 min. Protein was then transferred to a sheet of nitrocellulose using a Transphor cell (Hoefer) at 1 amp for 3 to 4 h in Towbin buffer at 4°C. Gels were removed and stained in Coomassie brilliant blue R-250 to check for complete protein transfer. Nitrocellulose transfer sheets were either stained for protein using Ponceau S, or incubated for several hours with 3% BSA, 20 mM Tris/HCl, 0.9% NaCl, 0.01% NaN_3 (pH 7.4) (BSA-Tris-saline) to block unreacted protein binding sites. Blots were then incubated for 1 h at 37°C or overnight at 4°C in 1% BSA-Tris-saline to which either 1:100 preimmune serum or 1:5000 antiserum had been added. Free antibody was then removed with four to five washes in 0.1% BSA-Tris-saline plus 0.5% Triton X-100 over a period of 1 h. Alkaline phosphatase conjugated to protein-A (Sigma) diluted 1:3000 in a solution of 0.1% BSA-Tris-saline plus 0.5% Triton X-100 was then added for 1 h. Blots were then washed thoroughly, first for 40 min in four to five changes of 100 mM Tris/HCl, 100 mM NaCl, 2 mM $MgCl_2$, 0.25% Triton X-100 (pH 7.5) and then similarly in 100 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$ (pH 9.5). For detection of antigen bands, nitrobluetetrazolium (0.34 mg ml^{-1}) and 5-bromo-4-chloro-3-indolylphosphate (0.17 mg ml^{-1}) were dissolved in 10 to 15 ml of the pH 9.5 wash buffer. The nitrocellulose blots were placed in this development solution for 15 min in the dark at which time the reaction was stopped with 10 mM Tris/HCl, 1 mM EDTA (pH 7.5). Developed blots were stored dry or in 20 mM Tris/HCl, 5 mM EDTA (pH 9.5).

RESULTS

The fifth leaf was used to investigate effects of heat stress on NMT activity because this leaf contained 10-fold more gramine when it developed at 30°C/25°C than when it developed at 21°C/16°C (11).

Effect of Growth Temperature on *in Vivo* NMT Activity. Segments of Arimar fifth leaves grown at 30°C/25°C incorporated three to ten times more [^{14}C]formate label into indole alkaloids than did similar segments from leaves grown at 21°C/16°C (Table I). Similar results were obtained with the gramine-free cultivar Proctor, provided that the precursors AMI or MAMI were supplied (Table I). Because the ^{14}C-incorporation was measured at 21°C/16°C in all cases, these results imply that growth at supraoptimal temperatures increased the level of NMT activity present in leaf tissue. To test the validity of this inference, we developed a method for assaying NMT activity *in vitro*.

Characterization of *in Vitro* NMT Activity. First leaves, which synthesize gramine constitutively, were used as a convenient source of NMT activity for defining assay and storage conditions. Under the standard assay conditions, ^{14}C-methylation of both AMI and MAMI substrates was linear for 40 min (Fig. 1). A 30-min incubation was therefore routinely used. Analysis of the labeled alkaloid products showed that when AMI was the methyl acceptor, 90% of the ^{14}C was present in MAMI, with the rest in gramine. When MAMI was the methyl acceptor, all radioactivity was in gramine. The NMT activity of leaf extracts was specific for gramine precursors; tryptamine was not methylated at all, and tyramine only slightly (Fig. 1). Furthermore, extracts prepared from wheat seedlings, a species lacking gramine, did not methylate supplied AMI or MAMI. The pH profiles for *N*-methylation of AMI and MAMI were similar between pH 7 and 10.5 (Fig. 2). Maximal catalytic activity with both substrates occurred close to pH 9.0. Sodium carbonate/bicarbonate and K-phosphate were found to be inhibitory; activities towards AMI and MAMI were affected equally (not shown). NMT activity was

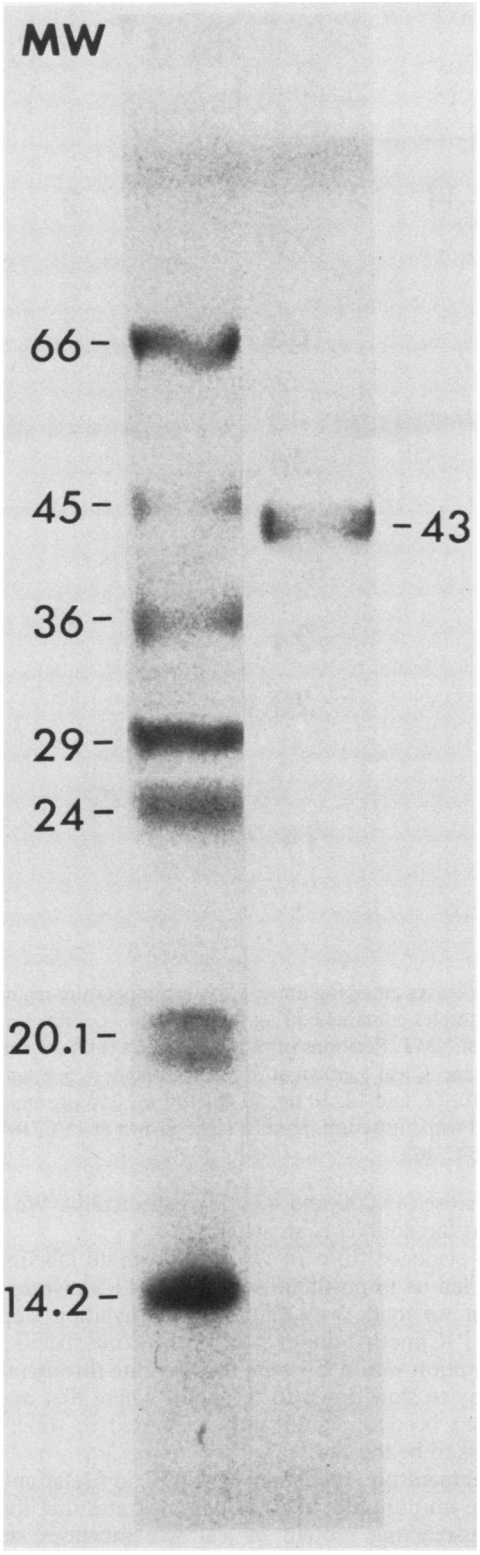

FIG. 4. SDS-PAGE of purified NMT protein and mol wt markers (kD). The NMT sample was from the second DEAE HPLC step (Table III). Acrylamide concentration was 13%.

quite stable in leaf extracts held at 4°C; after 4 d, activity with AMI or MAMI substrates was about 75% of original. However, a single freeze-thaw cycle reduced both activities to half that of original.

Effect of Growth Temperature on *in Vitro* NMT Activity. For the fifth leaf of Arimar and Proctor, NMT activity towards both AMI and MAMI increased as growth temperature was increased between 15°C/10°C and 35°C/30°C (Fig. 3); NMT activities at these extremes differed by a factor of about 8 for Arimar, about 20 for Proctor. In contrast, NMT activities in the second leaf remained low at all temperatures, consistent with the failure of the second leaf to accumulate alkaloids in comparable experiments (11). NMT activities in Figure 3 are expressed on a fresh weight basis, but results for leaf 5 expressed as specific activity are very similar because soluble protein levels were 20 to 22 mg/g fresh weight at all growth temperatures. For both cultivars, growth was markedly poorer at the two higher temperature regimes.

The plants of Figure 3 had been grown in the various temperature regimes for at least 12 d. To determine whether shorter intervals of heat stress would elicit an increase in NMT activity, plants were grown at 15°C/10°C and exposed to heat stress for various times (Table II). None of the shorter stress exposures elicited more than one-third of the NMT activity found after a 14-d exposure.

Purification and Characterization of NMT Protein. NMT protein was purified from dark-grown first leaves; Table III summarizes typical results. Throughout purification, NMT activity towards AMI and MAMI remained in the same ratio (~1:0.7). On gel filtration under nondenaturing conditions (not shown), NMT activity eluted very close to the ovalbumin standard (mol wt 45,000). SDS-PAGE (Fig. 4) indicated a mol wt of ~43,000. Purified NMT protein often, but not always, ran as a doublet on 13% gels.

Effect of Growth Temperature on NMT Protein Level. In an experiment similar to that of Figure 3, fifth leaf extracts were assayed for NMT activity and analyzed by immunoblotting using antiserum directed against NMT protein. NMT activity levels were comparable to those shown in Figure 3. Because data for Arimar and Proctor were similar, only data for Arimar are given. Immunoblots of crude extracts of fifth leaves grown at 15°C/10°C showed no immunologically detectable bands (Fig. 5A), or very weak ones (Fig. 5B). Extracts of fifth leaves grown at 21°C/16°C, 30°C/25°C and 35°C/30°C gave progressively stronger bands migrating to the same position as purified NMT protein (Fig. 5A). This steady increase in cross-reacting material parallels the behavior of enzyme activity (Fig. 3). Consistent with the lack of NMT induction and alkaloid synthesis in the second leaf, extracts of this leaf showed very weak immunologically detectable bands, whether the plants had been grown at 15°C/10°C or 35°C/30°C (Fig. 5B).

DISCUSSION

Coordinate Regulation of Steps in Gramine Biosynthesis. The results demonstrate that NMT activity specific to the gramine pathway is induced in growing leaves, but not in mature leaves, by prolonged exposure to high temperature stress. This mirrors the pattern of induction of overall gramine pathway activity (11). Because the intermediates AMI and MAMI do not accumulate, the *N*-methylations of gramine synthesis can never be rate limiting in the overall pathway (11, 22). These observations suggest that NMT activity is regulated coordinately with the activity of the rate-limiting step; nothing is known about this step save that it lies between tryptophan and AMI (11). It is interesting that the cultivar Proctor shows normal NMT induction even though it has an early lesion in the gramine pathway, and is alkaloid free. This establishes that the NMT induction mechanism is independent of the alkaloid products of the pathway.

Is There a Single NMT Enzyme? Although we cannot exclude the possiblity that there are two physically similar NMT enzymes specific for AMI or MAMI substrates, three lines of evidence point to a single enzyme. First, activities towards AMI and

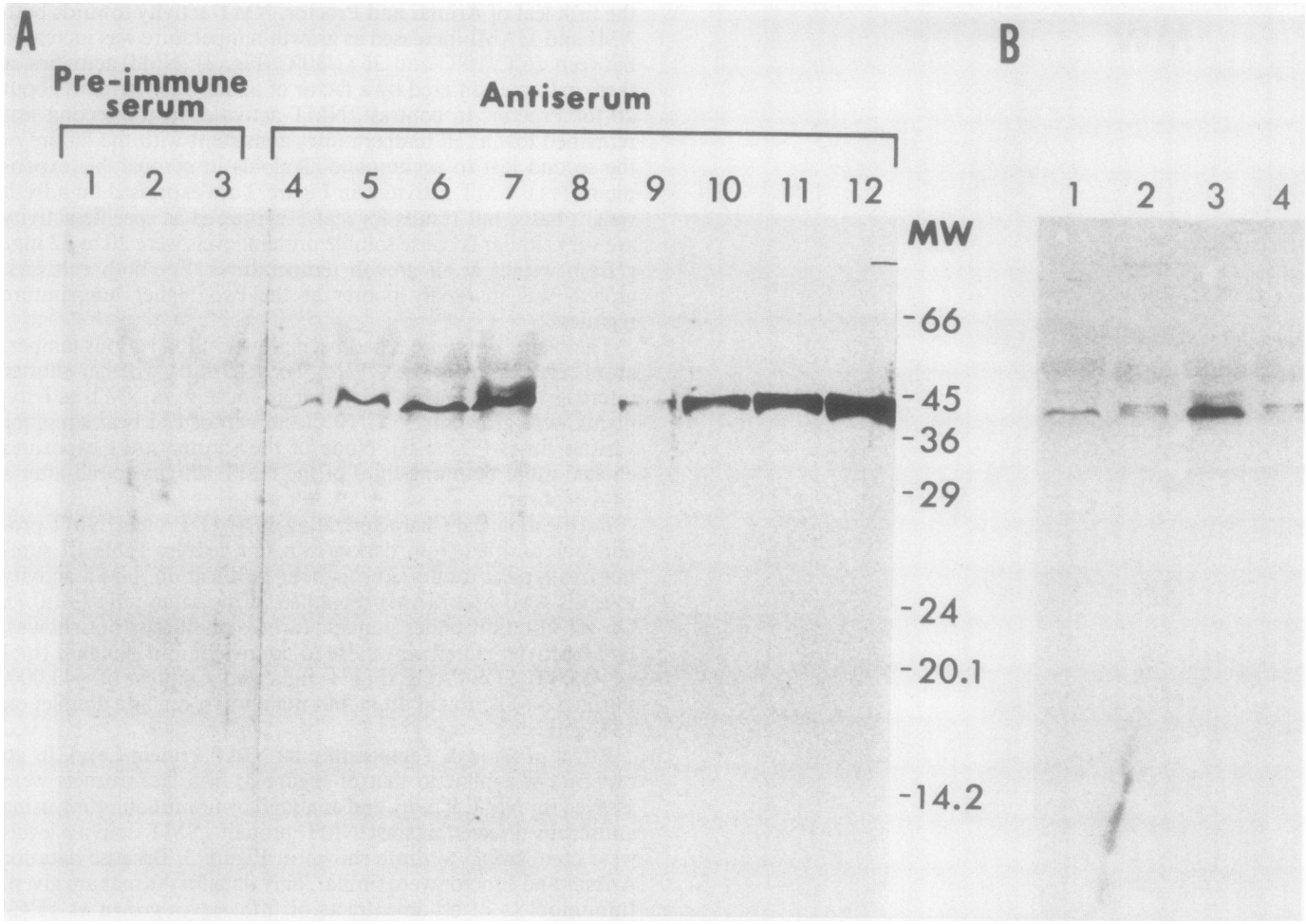

FIG. 5. Immunoblot analysis of NMT polypeptide levels in crude extracts of Arimar leaves emerging under various temperature regimes. Plants were grown for 10 d at 15°C/10°C before transfer to other regimes. Lanes with leaf samples contained 50 μg total protein. A, Fifth leaf samples. Lanes 1 to 3 were probed with preimmune serum; lanes 4 to 12, with antiserum against NMT. Positions of mol wt markers (kD) are indicated on the right. Lane 1, 250 ng of purified NMT protein; lane 2, leaf 5 grown at 15°C/10°C; lane 3, leaf 5 grown at 35°C/30°C; lanes 4, 5, 6, and 7, leaf 5 grown at 15°C/10°C, 21°C/16°C, 30°C/25°C, and 35°C/30°C, respectively; lanes 8, 9, 10, 11, and 12, 10 ng, 50 ng, 100 ng, 250 ng, and 500 ng of purified NMT protein, respectively. B, Leaf 2 and leaf 5 samples. All lanes were probed with antiserum. Lane 1, leaf 5 grown at 15°C/10°C; lane 2, leaf 2 grown at 15°C/10°C; lane 3, leaf 5 grown at 35°C/30°C; lane 4, leaf 2 grown at 35°C/30°C.

MAMI were not resolved by any of the separation methods applied, and the activities co-purified in a constant ratio of ~1:0.7 (Table III). Second, approximately the same ratio of 1:0.7 was found for NMT activities in crude leaf extracts, regardless of genotype, growth temperature, and leaf position. Also, leaf age was found not to affect the ratio in experiments with first leaf samples between 1 and 5 weeks old (not shown). Last, the two activities showed the same pH optima, the same sensitivity to inhibition by buffers, and the same stability to storage at 4°C or freezing/thawing. Criteria similar to these have established that there are separate NMT enzymes for the sequential *N*-methylations of tyramine in barley roots (16) and of tryptamine in *Phalaris* shoots (15). We therefore hypothesize that there is a single NMT enzyme that catalyzes both the methylations of gramine biosynthesis. Genetic evidence is consistent with this (T. J. Leland, R. Grumet, and A. D. Hanson, unpublished data).

Although purified NMT protein was often resolved into a doublet on SDS gels, it is unlikely that this doublet results from the presence of separate NMT enzymes, since the staining intensity of the lower band varied among experiments whereas the activities towards AMI and MAMI did not. Dissociation of enzyme subunits is likewise improbable, because the mol wt estimates from gel filtration of native enzyme and SDS-PAGE were very close (45,000 and 43,000, respectively). We therefore suggest that the doublet is an artifact.

Indirect evidence from *in vivo* [^{14}C]formate labeling studies previously led us to postulate separate NMT enzymes (11). An assumption we made was that both methylation steps would draw on a [^{14}C]methyl donor pool of the same specific activity. This assumption would fail were the flux rate through the gramine pathway to slow down to the point where first and second methylations become significantly separated in time; we now suppose this to be the case.

High-Temperature Induction of NMT in Relation to Heat Shock. The immunoblot analysis demonstrates that the level of NMT cross-reacting protein in leaf five increases steadily as growth temperature is raised and so implies that the heat-induced increase in NMT activity is due, at least principally, to an increase in enzyme level. Such a heat-induced increase in the abundance of a protein bears some resemblance to the heat-shock response (1, 13). However, NMT induction differs from induction of HSPs in several ways. First, synthesis of HSPs is strongly induced by brief (minutes to hours) exposure to high temperature and declines during prolonged exposure (4, 13), whereas NMT induction apparently requires several days exposure for full expression. Second, the heat-shock response occurs in almost all tissues

of the plant (5), but NMT induction is restricted to growing leaves. Third, the NMT protein is highly specific to barley, unlike HSPs, at least some of which show close homologies among all living organisms (21). Last, HSP induction generally has a sharper temperature threshold than does induction of NMT activity.

The conditions for induction of NMT activity and gramine accumulation—prolonged exposure to high temperature during leaf growth—imply that there is a window in leaf development when high temperature can enhance the expression of an NMT gene, and perhaps also of a gene governing conversion of tryptophan to AMI. The window coincides with the phase of cell division and elongation of the leaf. We speculate that this NMT induction response is representative of a special class of environmental regulation in plants: the eliciting of genetic information in a time-dependent way when chronic environmental stress is imposed on meristematic cells. We further speculate that the phenomenon of progressive adaptation of dividing cells in culture to long-term osmotic stress (10) is another example of this type of environmental control of plant function.

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